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DEVELOPMENT AND EVALUATION OF ADENO HTLV-III HYBRID VIRUS AND NON-CYTOPATHIC HTLV-III MUTANT FOR VACCINE USE

Annual Report

by

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Conditions were established and standardized for reproducibly infecting Cercopithecus with HIV-2 in the laboratory. Cercopithecus infected and vaccinated with HIV were monitored for an immune response on the basis of antigen-induced cellular proliferation and development of an antibody response detectable by Western blot assay. The anti-HIV antibody was also characterized for its ability to neutralize HIV virus. Wild Cercopithecus were tested for naturally occurring HIV infection and retroviruses isolated from five of these animals have been propagated in vitro.

After screening and two cycles of plaque purification, four recombinant HIV gp120/adenovirus 2 isolates were obtained that produced HIV envelope protein as determined by immunocytochemical staining using a rabbit antibody directed against gp120. The HIV gp120 sequences in one of these isolates were incorporated into the early region 1B rather than into the targeted early region 3.

Alternative methods for generating recombinant adenoviruses that would simplify future

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SUMMARY

The immune response of Cercopithecus to HIV has been monitored in infected and vaccinated animals. Anti-HIV antibody was characterized for its ability to neutralize HIV virus and its reactivity in the HIV Western blot assay. Antigen-induced cellular proliferation was also studied. Preliminary experiments to establish protocols for measuring cytotoxic T cell responses were conducted. Standardization of the conditions leading to reproducible infection of Cercopithecus with HIV established that this species could be infected most reproducibly by intravenous administration of HIV-2. Cercopithecus were tested for "natural" infection with HIV. Retrovirus from 5 Cercopithecus have been propagated in vitro.

Screening for recombinant HIV gp120/adenovirus 2 continued in an attempt to obtain a plaque-purified isolate that produced significant quantities of the HIV envelope protein. After two cycles of plaque purification, only 50% of three purified virus isolates were recombinants that contained gp120 sequences detectable by dot blot hybridization. Of the six plaque-purified recombinant viruses tested, four produced HIV envelope protein as determined by immunocytochemical staining using a rabbit antibody directed against gp120. Southern blotting of viral DNA extracted from cells infected with these recombinants demonstrated that, in one of these isolates, the HIV gp120 sequences were incorporated into the early region 1B at the left end of the adenoviral genome rather than into the targeted early region 3 near the right end of the genome. To provide an alternative means for generation of recombinant adenoviruses, vectors were constructed that contained the XbaI C fragment from the right end of the adenovirus genome. Cells transfected with a 31.9 kb AseI DNA fragment extending from the left end of the adenovirus 2 genome and the vector containing the rightmost XbaI DNA fragment plus HIV envelope sequences should produce few or no wild type progeny viruses, thus simplifying future screening for recombinant adenoviruses.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals", prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

The investigator(s) have abided by the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules (April 1982) and the Administrative Practices Supplements.

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BODY OF REPORT

A. Statement of Problem:

Acquired immunodeficiency disease syndrome (AIDS) was initially recognized as a separate disease in 1981. Results from research groups in France and the United States determined that a previously unknown retrovirus, the human immunodeficiency virus (HIV) was the primary etiological agent of AIDS. Two types of this retrovirus have been isolated; HIV-1 from AIDS patients in the United States, Europe and Central Africa (1,2) and HIV-2 from AIDS patients mainly in West Africa (3,4).

AIDS is a highly lethal disease and presents a serious medical, social and economic problem of global dimensions. In 1987, the World Health Organization estimated the total number of AIDS cases worldwide to be 100,000 with an additional 5 to 10 million asymptomatic HIV-infected individuals (5). In the United States alone, more than 82,764 cases had been reported by December, 1988, with a mortality rate of greater than 50% (6). James Curron of the U.S. Center for Disease Control, Atlanta, Georgia, estimates that, within 5 years, AIDS will be the leading cause of death among young and middle-aged men (7). In the past, the major mode of AIDS transmission was considered to be through homophilic sexual practices and via contaminated needles associated with drug abuse. However, evidence is accumulating that heterosexual transmission with attendant maternal-fetal infection is, likewise, an important mode of HIV transmission, thus, putting the entire population "at risk". The spread of AIDS into the general population underscores the urgency of developing an effective vaccine against HIV as immunoprophylaxis appears to be a rational and effective means to control the spread of this disease.

Up to 100% of patients with AIDS and pre-AIDS syndrome have anti-HIV antibody in their sera (8), suggesting that anti-HIV antibody alone is not sufficient to protect patients from development of AIDS. The aim of this proposal is to study the role which T cell mediated immunity and anti-HIV antibody play in preventing or ameliorating HIV infection and to use this information for the development of an effective HIV vaccine.

B. Background:

AIDS is a recently identified disease characterized by increasing deficiencies in the body's cell mediated immune response in previously normal patients

(8). A reduction in the number of helper T lymphocytes (OKT4+) is usually involved and is accompanied by multiple opportunistic infectious and/or malignancies (9,10). A syndrome designated AIDS related complex (ARC) has been identified in groups at risk. The dominant clinical expression in ARC is unexplained chronic lymphadenopathy or leukopenia involving a reduction in OKT4+ cells. Minor cutaneous infections, diarrhea, weight loss and fever may be associated with ARC (8).

Two subtypes of virus have been identified as etiologic agents of AIDS. In North America, Europe and Central Africa, AIDS is associated with HIV-1 infection (1,2). Recently, a retrovirus designated HIV-2 has been isolated from AIDS patients and healthy West Africans (3,11). Nucleic acid sequence analysis of HIV-1, HIV-2 and simian immunodeficiency virus (SIV) reveals 75% homology between HIV-2 and SIV and 45% homology between HIV-2 and HIV-1 (12,13). HIV-1 and HIV-2 serological cross reactivity is restricted to core proteins. HIV-2 positive sera cross react with all SIV proteins, including envelope (3). HIV-1 and HIV-2 appear to differ in their pathogenicity. HIV-1 infection progresses to AIDS in at least 25% of infected individuals within 5 years following seroconversion (14). In West Africa, the number of AIDS cases is relatively low in HIV-2 seropositive patients.

Chimpanzee is the only non-human primate known to be susceptible to infection with HIV (15-17). Infected chimpanzees develop a transient severe lymphoadenopathy. Virus can be recovered from peripheral blood lymphocytes and bone marrow but not the plasma, saliva or cerebralspinal fluid (18). Although HIV-inoculated chimps remain persistently infected, a persistent decrease in T4+ cells does not develop and the chimpanzees remain clinically well (19).

Preliminary experiments by Dr. Daniel Zagury indicated that African Green monkeys (Cercopithecus) infected with HIV virus by intraperitoneal (IP) injection develop viral infection. Some animals showed evidence of infection as detected by reverse transcriptase activity, ability to transmit HIV infection to peripheral blood lymphocytes and the presence of HIV genome in their peripheral blood lymphocytes. Some monkeys had a serum antibody response to HIV. Because not all of infected animals could be shown to be infected, an alternate model, HIV-2 infection of Cercopithecus has been developed.

In order to be successful, a vaccine must elicit an immune response that is neutralizing or protective. Although anti-HIV antibodies may neutralize at the

intial stages of viremia, they do not appear to be protective since patients with high titers of anti-HIV antibodies develop AIDS (20). Cytotoxic T cells can play an important role in host defense against viral infectious (21) and vaccination leading to cell mediated immunity to HIV may be desirable because cytotoxic T cells could protect from further cell to cell viral spread. HIV specific T lymphocytes in seropositive individuals have been demonstrated (22-25), but the role they play in AIDS is not known. T cell receptors present a heterodimeric reactivity that is directed against both self major histocompatibility antigens and processed antigenic determinants. In contrast to antibodies, which may be serotype specific, cytotoxic T cells exhibit broader antigenic specificities. This accounts for cross reactions observed by cytotoxic T cells of autologous targets infected by different strains of virus with serologically distinct influenza virus (26,27). Thus a vaccine inducing cytotoxic T cells against one subtype of HIV may destroy cells infected with other HIV subtypes.

The objective in the development of a HIV vaccine is to produce, with minimal side effects, an immune response of long duration in the host that will provide effective protection against viral infection and the subsequent onset of clinical AIDS. The worldwide prevalance of AIDS also makes it important that such a vaccine be easily administered and relatively inexpensive to produce.

The majority of viral vaccines now in use are preparations of either live, attenuated or killed virus. Concerns for reversion to virulence and the risk of reactivation of intact virions or proviral DNA make these approaches to a HIV vaccine less desirable. At present, subunit vaccines utilizing immunogenic virus peptides or proteins derived from purified virions or by recombinant DNA technology appear to provide the best approach to development of a safe and effective vaccine. These subunit components may be administered in association with adjuvants or carriers, incorporated into artifical "membranes" or delivered by means of recombinant viruses. Several recombinant vaccinia-HIV viruses have been developed and tested in non-human primates (28-32) and humans (33). Chimpanzees vaccinated with vaccinia-HIV demonstrate very low titers of neutralizing antibodies (28). Therefore, the development of an alternate HIV recombinant vaccine may prove valuable. For a variety of reasons outlined below, we have proposed to utilize human recombinant (r) adenovirus (Ad) as a vector for expression of the HIV glycoprotein, gp120.

Bivalent immunization with live, attenuated Ad type 4 and type 7 has proven to be both safe and effec-

tive in military recruits over a period of 20 years (34-36). This vaccine, which is administered orally in enteric-coated capsules, liberates virus into the intestine where a subclinical infection is established that confers a high degree of immunity. Successful immunization with Ad types 1, 2 and 5 by this route have also been demonstrated (37). The fact that administration of live vaccines by the gastrointestinal route may facilitate spread of the Ad (38) may be advantageous in immunizing against HIV if a suitable, relatively non-pathogenic strain of adenovirus is used as vector. The majority of Ad infections result in self-limiting and short-lived clinical manifestations although prolonged asymptomatic or latent infections may occur (39). It is possible that reactivation of latent adenovirus, as has been proposed to occasionally occur in rubella infections (40), may provide a mechanism for a natural HIV "booster" immunization in the case of the rAd vaccine.

Ad structural proteins are synthesized in large quantities during infection and at least 80% of viral hexon, penton and fiber are not incorporated into progeny virus but remain in the infected cell in the form of readily soluble multimers (41). Theoretically, exogenous DNA sequences stably integrated into the Ad genome under the control of the major late promoter might also be expressed at a high level to provide a source of immunogen.

Ad is very stable and can tolerate temperatures of 4-36^{o}C and pH 5-9 with minimal loss of infectivity, thus, alleviating many problems associated with vaccine transport and storage especially in under-developed countries.

The technology necessary to propagate large quantities of Ad to produce an enteric-coated vaccine is presently available so that production of a rAd vaccine should not require the development of new manufacturing processes.

This project is designed to investigate the feasibility of using infectious Ad to produce gp120 or other HIV immunogen and to determine whether these HIV antigens would be presented in such a way as to elicit protective immunity in the host.

C. Rationale

Chimpanzees are the only non-human primate whose ability to be infected with HIV is well documented. We feel that further characterizing the Cercopithecus monkey as an alternative model is important since the chimpanzee model has several limitations. Chimpanzees

are an endangered species and difficult to obtain in large enough numbers to conduct a vaccination protocol. They are expensive to maintain and although chimpanzees can be chronically infected with HIV, they remain clinically well (18). Cercopithecus may prove to be a useful model since enough animals to conduct a vaccination trial can be obtained and maintained at a relatively low cost. Preliminary experiments with Cercopithecus established that this species can be infected with HIV-1. Future experimentation revealed that using the original protocol, not all Cercopithecus responded with full evidence of infection and an anti-HIV immune response. Full evidence of infection was defined as reverse transcriptase (RT) activity in supernatants of peripheral blood lymphocytes (PBL), immunofluorescent staining of PHA activated PBL, HIV transmission to T cells and an intergrated HIV genomic pattern Southern blot DNA hybridization. Experiments to determine the protocol necessary to infect 100% of Cercopithecus were begun. Parameters studied included:

a) Varying the HIV strain: HIV-1 and HIV-2 were tested.

b) Varying the route of infection: Intravenous (IV) and intraperitoneal (IP) routes were investigated.

c) Varying the number and dose of virus.

We have not conducted any studies with HTLV-III-X10-1 mutant virus since vaccination with live mutant HIV virus is not practical due to the possibility of reversion to virulence. Therefore, the immune response to recombinant vaccinia-HIV virus containing gene sequences to HIV gp160 has been studied. Because T cells can play an important role in host defense against viral infections (21), it is important to investigate T cell mediated immunity in vaccinated or infected Cercopithecus. Both T cell mediated lysis of infected targets and antigen induced cellular proliferation have been investigated. Serological studies have also been conducted.

Retroviruses-related to HIV-1 and HIV-2 have been isolated from various non-human primate species, including rhesus monkeys (42), Mangabey monkeys (42), African green monkeys (43) and Mandrills (44). Because the ancestors of HIV-1 and HIV-2 probably evolved from a lentivirus infecting primates in Africa, studies of retrovirus from wild Cercopithecus in Zaire should also help to delineate the evolutionary relationship for human and non-human primate retroviruses.

The rAd for vaccine purposes is obtained through

recombination between homologous regions of viral DNA and a plasmid construct containing HIV sequences. Ad type 2 (Ad2) was selected as the vector because this serotype has been extensively characterized on the DNA, RNA and protein level and is easily propagated in the laboratory. The viral early region 3 (E3), which is not essential for Ad2 replication in vitro, is replaced with the desired HIV sequences. Nucleotide sequences encoding gp120, the major external glycoprotein exposed on the surface of HIV, were used in the initial construct of the rAd plasmid vector. Gp120 was selected because this surface glycoprotein is the most immunogenic portion of the virus (45) and is essential for infectivity. Precedence has shown that immunization with retroviral glycoprotein(s) elicit both neutralizing and cytotoxic antibodies. The HIV gp120 DNA fragment used to construct the rAd vector extends from a SspI site 65 base pairs (bp) upstream of the initial ATG of the envelope coding sequence to a second SspI site at nucleotide 7567 of HIV strain HXB2. This fragment includes 87% of the gp120 coding sequence plus the 30 amino acid (aa) leader. Because the HIV transmembrane protein, gp41, may also be a potential immunogen, a second vector which includes the entire HIV env gp160 sequence has also been constructed. In all constructs, the gp120 or gp160 coding sequences are flanked 5′ by the Ad major late promoter including the tripartite leader and 3′ by an Ad polyadenylation signal and VA RNA coding region.

Chimpanzees have been the sole animal model in which to conduct valid tests of potential vaccines because these animals are susceptible to HIV infection, react immunologically, and simulate the lymphoadenopathy syndrome. However, the proposed rAd vaccine cannot be evaluated in chimpanzees because human Ad do not replicate well, if at all, in monkey cells, and, virus replication is essential for expression of the HIV sequences. This block to Ad replication in simian cells appears to occur at the RNA splicing level (46) and is overcome by the carboxy terminus of the simian virus (SV) 40 T antigen (Ag)(47). Therefore, the initial rAd vaccine construct contains approximately 500 bp of 3′ SV40 T Ag coding sequence plus 200 bp of early SV40 DNA driven by the SV40 promoter. This fragment with SalI sites on either end can easily be removed and the plasmid religated to provide a construct minus SV40 sequence for the human vaccine. The presence of SV40 sequences also provides a means to select recombinant Ad2 progeny virus based upon plaque size. Theoretically, rAd containing the SV40 fragment should replicate more efficiently and rapidly on the CV-1 African green monkey kidney cell line and produce larger plaques.

To facilitate homologous recombination into the Ad E3 region, the vaccine plasmid construct contains flanking Ad sequences with 1051 bp of E3 and protein pVIII coding region upstream and 1513 bp of E3 and fiber coding region downstream of the gp120-SV40 sequences.

Because Ad2 is endemic with neutralizing antibody found in 60% of the U.S. population (48), it is essential to change the Ad serotype in order to develop an effective human vaccine. Two approaches can be taken: 1) use a more rare and relatively nonpathogenic Ad serotype to construct the rAd vaccine virus or 2) exchange the Ad2 sequences encoding the fiber, which is primarily responsible for type-specific neutralizing antibody (49), with that of a rare strain. Therefore, the final live rAd vaccine virus will consist of HIV gp120 sequences expressed from the Ad major late promoter inserted in the E3 region of a modified Ad2 or non-Ad2 virus vector.

D. Experimental Methods:

1. Facilities:

Two units of Cercopithecus are maintained, the first at the Primatology Center of CNS in Villejuir, Paris (directed by Dr. Pierre Dubouch) and the second in Kinshaw, Zaire (directed by Mrs. Delphi Messinger, an American Zoologist).

Newly acquired Cercopithecus are anesthetized with ketamine, T-B tested in the left eyefold and given an overall physical exam. Periodical checks are made for parasites and animals are wormed as needed. Animals are provided access to water and are fed a cake of wheat, corn and soy flour with peanuts, milk, millet, bananas, and other fruits, nuts and various greens.

A field laboratory at Lubutu, Zaire has been established in July 1988. Serum and cell samples from non-endangered Cercopithecus species killed or trapped in this area are analyzed for evidence of retrovirus infection. In addition to Ms. Messinger and Dr. Daniel Zagury, the Lubutu Laboratory is staffed by 2 technicians, a Zairian doctor (Dr. Ngoi), his nurse, one Zairian veterinarian and one driver. Plasma, sera, spleen and PBL are frozen in liquid nitrogen the same day collected. Samples are taken from Cercopithecus killed by inhabitants for food if it can be established that the Cercopithecus are less than 6 hours hours dead. In order to capture wild retrovirus-infected Cercopithecus, animals were first anathesized and blood samples taken. Quick serological tests were performed to determine cross reaction with HIV-1 or HIV-2. The animal is captured if the test is positive or released into the wild if negative.

2. Reverse Transcriptase

Culture supernatant (1 ml) was centrifuged at 1300 x g for 10 minutes. The supernatant was removed and centrifuged at 40,000 x g for 2 hours to pellet virus. The virus pellet was resuspended on ice in 20 ul of buffer containing 0.05M Tris-HCl pH 7.5, 0.3M KCl, 0.3% Triton X-100. To this was added 40 ul of buffer containing 0.07M Tris-HCl pH 8.3, 0.014M $MgCl_2$, 0.03M KCl, 0.0014 M DTT, 15 ug/ml polyadenylic acid, 15 ug/ml oligothymidylic acid (dT) 12-18, and 3 uCi $_3$H-TTP (46 Ci/mmol). Samples were incubated at 37°C for 1 hour and then the reaction was stopped on ice by addition of 0.1 ml of 0.1M sodium pyrophosphate solution in 5% TCA, 250 ul of 0.5 mg/ml of yeast nucleic acid, and 3.5 ml of 20% TCA. Samples were kept 15 minutes at 4'C and then spotted onto a Whatman No. 3 disk. The radioactivity incorporated in trichloracetic-precipitable material was measured in a scintillation counter; and the results were expressed in counts per minute per ml of culture medium.

3. Virus Inoculation

Virus inoculation protocols varied and are described in results. The inoculata were cell-free virus or autologous infected cells. The virus strain HIV-2 NIH-DZ was generously provided by R.C. Gallo. The inoculation of autologous infected cells involves in vitro infection of 3 day PHA-activated PBL, treated for 20 min with polybrene (2 mg/ml). The virus was added to the cell-pellet containing 10^7 cells for 1 hour at ambient temperature and then 5 ml of RPMI 1640 plus 15% FCS (fetal calf serum) containing 0.2% of interleukine 2 (recombinant), 10% hydrocortisone and 1% sheep anti-human interferon serum were added slowly above the cell-virus mixture. Reverse transcriptase activity was measured twice weekly until a peak of reverse transcriptase activity was obtained. The cells were washed, counted in trypan blue solution and resuspended in 2 ml of RPMI with 5% FCS for inoculation. Generally, the highest RT level was obtained after 7-10 days (50).

4. Virus Isolation

Heparinized blood samples were centrifuged to remove the plasma. Ficoll-hypaque gradient purified PBLs were stimulated with Phythohemagglutinin (PHA) for 3 days in coculture with normal human PBL, in RPMI 1640 plus 15% FCS supplemented with 0,2% IL-2, 10% hydrocortisone and 1% anti-human alpha interferon sheep serum. The medium was renewed twice a week and the reverse transcriptase activity measured. Positive supernatants were used to infect activated human PBLs by transmission (51), in

order to obtain a high number of infected cells and to have sufficient material for DNA extraction.

5. Immunoassays

Anti-HIV antibody was measured using commercially available ELISA assays (Abbot, Chicago, IL) or Spot Test (Organix). Positive sera were confirmed by Western Blot assay using HIV-1 strips (Pasteur) or HIV-2 strips (DuPont, Wilmington DE). Commercial ELISA assay (Abbott) was used to detect HIV-1^{125} p24 antigen. Neutralization antibody assays were performed according to methods of Robert-Guroff (52). H9 cells were cultured with a titered suspension of HIV that had been preincubated with the test serum for one hour at 4^{o} and then 30 minutes at room temperature. HIV infection was measured by determining the percentage of positive cells 5 or 6 days later with indirect immunofluoresence (IFA). As controls for viral infection, virus was incubated with medium alone in the absence of serum and/or incubated with serum negative for anti-HIV antibodies. Both these controls gave similar results of a range of 50 to 60% of infected cells by IFA. The control for positive neutralization was a human serum positive for HIV antibody which blocked all infection (<1% infection by IFA).

6. Indirect Immunofluorescence Assay (IFA)

Indirect immunofluorescence assay was performed as already described (53). Cells were washed in 1X phosphate buffered saline, spotted on a slide, dried, and fixed with acetone-methanol (1:1) for 15 minutes at room temperature. Ten microliters of human polyclonal antiserum to HIV-1 (diluted 1:50 with phosphate buffered saline) were applied and the cells were incubated for 55 minutes at 37^{o}C. After washing (20 minutes), the fluorescein conjugated anti-human serum (1:100) was added, and the cells were incubated for 30 minutes at room temperature and then washed. A blank control contained no polyclonal human serum. H9 cells and HIV infected H9 cells (H9-HIV) were used as the negative and positive controls, respectively.

7. Lymphocyte Phenotyping

CD_4 and CD_8 T cell antigens were detected respectively by OKT_4 and OKT_8 monoclonal antibodies using the IFA or specific rosetting technique.

8. Cells and Viruses

293 cells, an Ad5 transformed human embryonic kidney line (54) and HeLa cells (ATCC CCL2) were maintained as monolayers in Dulbecco's modified minimal

essential medium (DMEM) with 1 gm/L glucose (GIBCO Laboratories) supplemented with 10% fetal bovine serum (GIBCO) and 0.1 mM MEM non-essential amino acids (GIBCO) at 37°C in a humidified, 7% CO_2 atmosphere. Cells were passaged by dissociation with 0.05% trypsin and 0.53 mM EDTA. To facilitate analysis of gp120 enveope protein synthesis during the course of infection with the recombinant Ad viruses, these cell lines were adapted for growth in reduced serum (2% fetal bovine serum; 293 cells) or serum-free (HeLa cells) QBSF-51 medium (Quality Biologicals).

Stocks of wild type Ad2 (ATCC VR846) and recombinant HIVgp120/Ad2 viruses were propagated in HeLa cells. Infected cells were disrupted to release intracellular virus by three cycles of freezing and thawing and the cell-free supernatant from a 30 min, 2500 xg centrifugation of disrupted cells plus culture medium was used as virus stocks. Wild type Ad2 and recombinant Ad2 were plaqued on 293 cell monolayers. Virus dilutions were prepared in phosphate buffered saline (PBS) with 2% heat-inactivated fetal bovine serum and absorbed to PBS-washed cell monolayers for 90-120 minutes at 37°C. Infected cells were overlayed with modified 2X Eagle medium (GIBCO) containing 4% heat-inactivated fetal bovine serum and 0.7% Noble agar (Difco Laboratories) supplemented with 10% tryptose phosphate (GIBCO) and 25 mM $MgCl_2$. Additional plaque assay medium was added at 3 day intervals post-infection (PI) with 0.02% neutral red (GIBCO) incorporated into the final overlay for plaque visualization. Plaques were scored at 7-8 days PI.

9. <u>DNA Transfection and Generation of Recombinant Adenoviruses</u>

Two methods were used to transfect 293 or HeLa cells with Ad2 DNA and plasmids containing HIV sequences. For the standard calcium phosphate procedure (55,56), cell monolayers at approximately two-thirds confluency were exposed to $CaPO_4$-DNA precipitates at a concentration of 2 ug per 35 mm dish for a period of 6 hours. In the second procedure (57), confluent monolayers of 293 cells in 35 mm dishes were in contact with a mixture of 2-4 ug DNA and 10 ug Lipofectin Reagent (BRL Life Technologies) in Opti-MEM I medium (GIBCO) for 8 hours. The DNA-containing medium was removed and replaced with fresh cell culture medium. The cells were monitored microscopically over a period of 1-2 weeks for development of distinctive cytopathology indicative of infection with adenovirus. If viral cytopathology was not observed, a blind passage was carried out in which the transfected cells were scraped from the surface of the culture dish and disrupted by alternative cycles of

freezing and thawing. The cell-free supernatant (2500 xg centrifugation; 30 min) was used to infect 293 cell monolayers that were observed for Ad viral cytopathology for an additional two weeks.

10. Plamid DNA Manipulations

Bacterial plasmid DNA preparations, enzyme digestions and agarose gel electrophoresis were carried out by standard techniques (58,59). Plasmid DNA was used to transform Escherichia coli (E. coli) DH5 cells. Ampicillin-resistant transformants were screened on the basis of size and restriction endonuclease digestion pattern. Restriction endonucleases were used according to the manufacturer's recommendations. The plasmid -actin 2000 containing 2108 base pais of -actin gene sequences cloned into the HindIII site of pBR322 was kindly provided by Steve Hughes (NCI-FCRF, Frederick, MD). Ad2 DNA was purchased from Life Technologies-BRL (Gaitherbsurg, MD).

11. DNA Probes

The DNA probe corresponding to HXB2 HIV gp120 nucleotides 6159-7568 was derived by BamHI digestion of plasmid pAD.MLPgp120. The probe corresponding to SV40 T Ag sequences was obtained by digesting plasmid pAD.ENV.ND1 with SalI. Adenovirus fragments used as probes were derived from EcoRI digestion of Ad2 DNA. Following digestion with the appropriate restriction enzyme, the DNA fragments were electrophoretically separated on 1% agarose gels and the desired fragment was recovered by electroelution.

12. Screening for Recombinant Adenoviruses

For screening, HeLa or 293 cells grown in 96 1/2-well plates (Corning Glass Works) were infected with: serial dilutions of wild type and recombinant virus mixtures; cells infected with the wild type and recombiant virus mixtures diluted to 0.8 infected cells per well; or single virus plaques obtained as plugs recovered from the agar overlaying cell monolayers on which wild type and recombinant virus mixtures had been plaqued. Blotted cell extracts were screened for the presence of HIV gp120 sequences by hybridization to the gp120 DNA probe labeled by random primed (60) incorporation of digoxigenin-labeled deoxyuridine-triphosphate using the Genius Nonradioactive DNA Labeling and Detection Kit (Boehringer Mannheim). Hybridization was carried out overnight at 68^{o}C in 1.5X SSPE (0.27M NaC1, 15mM Na_2HPO_4 7H2), 1.5mM EDTA)/1.0% sodium dodecyl sulfate (SDS) with 0.5% BLOTTO (nonfat powdered milk) and 10%

dextran sulfate. Following hybridization, membranes were sequentially washed with 2X, 0.5X and 0.1X SSC (20X SSC = 3M NaCl/0.3M sodium citrate) plus 0.1% SDS at room temperature. DNA hybrids were detected by the Genius enzyme-linked immunoassay using anti-digoxigenin alkaline phosphatase conjugate and subsequent enzyme-catalyzed color reaction with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium salt.

13. Preparation and Analysis of Viral DNA

Low molecular weight viral DNA was isolated from Ad-infected 293 or HeLa cells using a modification of the method of Hirt (61) in which 0.5 mg/ml pronase was added to the SDS lysis solution. For Southern blotting (62), the DNA was digested with appropriate restriction enzymes, electrophoresed through 0.7% agarose gels and transferred to Hybond-ECL nitrocellulose membrane (Amersham) with 20X SSC. Blots were probed by hybridization to DNA fragements labeled with horseradish peroxidase by the method of Renz (63) using the ECL Gene Detection System (Amersham). Hybridizations were carried out in ECL hybridization buffer at 42°C overnight and membranes were washed first with 0.5X SSC plus 0.4% SDS and 36% urea followed by 2X SSC as specified in the ECl protocol. DNA hybrids were detected by enhanced chemiluminescence (64) of horseradish peroxidase-catalyzed oxidation of luminol using ECL Gene Detection System detection reagents.

14. RNA Isolation and Analysis

Total cellular RNA was extracted from Ad-infected cells lysed with 4M guanidinium (65) and isolated by centrifugation through a 5.7M CsCl step gradient (66). RNA dilutions were applied to Hybond ECL nitrocellulose membranes using a slot blot manifold. For Northern blotting, RNA samples were electrophoresed on 1.2% agarose formaldehyde gels (67), transferred to Hybond ECL nitrocellulose membranes with 20X SSC and hybridized to DNA probes labeled with horseradish peroxidase using the ECL Gene Detection System (Amersham).

15. Immunocytochemical Staining

Cells were grown in disposable 8-chamber glass tissue culture slides (Lab-Tek) pre-treated with 50% fetal boving serum. Ad virus infections were carried out as described above. Cells were fixed by the addition of one-half volume freshly prepared 3:1 methanol-glacial acetic acid to the culture medium; and, after aspiration of the medium-fixative, by immersion in -20°C acetone for 30 minutes. The primary antibodies used for immunoperoxidase staining reactions were:

goat anti-gp160 (Dupont) (1:500); rabbit anti-gp120 (1:75); mouse monoclonal anti-gp120 96-16 (1:50): mouse anti-env ascites fluid (1:2). Peroxidase-conjugated swine anti-rabbit immunoglobulins or rabbit anti-goat or anti-mouse immunoglobulins (Dako Corp.) were used as second antibody. Color was developed with 0.01M o-dianisidine or 3,3', 5,5'-tetramethylbenzidine and hydrogen peroxide.

E. Results and Discussion

1. Monkey Research

Cercopithecus were tested for "natural" infection with retrovirus using Abbott Elisa to detect serological reactivity to HIV-1 virus and Orgenix Spot Test to detect serological reactivity to peptides of the envelope gene of HIV-1 and HIV-2. Seventeen Cercopithecus tested positive in at least one of the assays (Table 1). Viral particles were isolated from activated PBL of 5 Cercopithecus (C2447, C2452, C2444, C2547 and C2485) cocultured with MOLT 4 (clone 8) cell line. High levels of RT activity (over 10 cpm/ml) was observed as well as a high percentage (20-60%) of cells positive for HIV signals as detected by IFA. Genomic analysis using PCR technique is positive for gag and env fragments with ^{32}PBH10 at a strengency characteristic of each provirus, indicating that there is no contamination.

Two captured Cercopithecus who were seropositive for HIV antibody developed clinical manifestations of AIDS. They developed skin disease with important weight loss and cachexia. Autopsy showed that they both presented septicemia, one due to normal colibaciullus and the other to Klebsiella pneumoniae. In addition, pathology showed complete aplasia of spleen white pulp and lymph nodes. Virus isolated from these monkeys will be cloned and genome sequenced.

Two series of 6 Cercopithecus were injected with anti-HIV-2 antibodies or normal immunoglobulin for 3 consecutive days. On the third day, all animals were challenged with HIV-2 (I.V) injection of 3.3×10^5 $TCID_{50}$). These animals will be monitored to determine if passive immunization protects Cercopithecus from HIV-2 infection.

Since Cercopithecus are more reproducibly infected with HIV-2, HIV-2 specific reagents including DNA probes, polymer se chain reaction (PCR) primers and antibodies (monoclonal and polyclonal) are being developed and characterized. Similar to HIV-chimpanzee model (18), HIV-2 infected Cercopithecus are persistently infected

with HIV-2 in the absence of clinical symptoms.

2. Selection and Isolation of Recombinant HIV-1/env Adenovirus 2

Initial screening of viral progeny obtained after transfection of 293 cells with wild type Ad2 DNA and the vaccine vector pAD.ENV (HIV gp120 sequences) or pAD.ENV.ND1 (HIV gp120 sequences plus SV40 T Ag sequences) yielded six independent isolates consisting of heterogeneous mixtures of wild-type Ad2 and recombinant virus. Employing a protocol similar to limited dilution cloning, 293 cells were enzymatically dispersed at 24 hours post-infection with 1 plaque-forming unit (PFU) per cell of each virus mixture. The infected cell suspension was used as inoculum for subconfluent 293 cell monolayers in 96-well plates at a multiplicity of 0.8 infected cells per well so that, theoretically, 80% of the wells should contain an Ad2-infected cell. The 96-well plates were microscopically monitored for the development of viral cytopathology and, subsequently, lysed with alkali and heat, blotted onto nylon membranes, and analyzed for the presence of recombinant adenoviral DNA containing gp120 sequences by hybridization of a digoxigenin-labeled. gp120 probe. This selection method yielded four isolates. Based upon the assumption that each infected cell well was derived from a single infected cell inoculum, the ratio of positive isolates per infected cell well ranged from 0.1 - 0.3% (Table 2). These numbers should reflect the percentage of recombinant Ad2 in the heterogeneous virus stocks.

To analyze the DNA of these putative recombinant adenoviruses, stocks of the J3A, G4A and two H6B isolates were prepared by infecting 293 cells with the culture medium saved from the hybridization-positive wells. Viral DNA was extracted from a portion of the infected cells (61), digested with the restriction enzymes, SalI and BamHI, electrophoresed through 1% agarose gel and transfered to nylon membrane for hybridization with a digoxigenin-labeled gp120 probe. An approximately 1800 base pair BamHI fragment hybridized to the gp120 probe in DNA extracted from cells infected with H6B isolate C (data not shown). This fragment is comparable in size to the 1400 base pair gp120 DNA coding sequences flanked by BamHI sites that is contained in the pre-vaccine vector pAD.ENV.ND1 used to produce the recombinant virus. DNA extracted from cells infected with the G4A isolate contained a BamHI fragment of approximately 12.5 kb and a SalI fragment of 10.5 kb that hybridized to the gp120 probe. Wild type Ad2 DNA digested with BamHI or SalI yields approximately 10.5 kb or 9.5 kb fragments, respectively, derived from the left end of the viral genome. Therefore, the hybridization results obtained

with the G4A isolate indicated the possibility that *in vivo* recombination between the vaccine vector and wild type Ad2 DNA might have occurred at a site other than the targeted Ad early region 3. No bands that hybridized to the gp120 probe were observed in DNA isolated from cells infected with the J3A isolate or the H6B-A isolate.

In addition, ten-fold dilutions of the culture medium from the hybridization-positive wells were used to infect 293 cells for plaque purification of the recombinant isolates G4A, J3A, H6B-A and H6B-C. The plaques were "picked" with a pipette and the agar plugs were used to infect 96-well plates of 293 cells. These cells were monitored microscopically. Upon observation of extensive CPE, the cells were lysed with alkali and heat and were blotted and hybridized to a digoxigenin-labeled gp120 probe. Three plaque-purified virus isolates were obtained from the G4A virus stock (Table 3); no plaque-purified isolates were obtained from the H6B-A, H6B-C, or J3A stocks in this initial plaque purification.

Monolayers of 293 cells in 6-well plates were infected in duplicate with 0.5 ml of a 10^{-2} dilution of culture medium from the three positive G4A wells (A11, G1, and G6) in the previous screen. When greater than 75% of the cells showed viral CPE, viral DNA was extracted by the method of Hirt (61). After digestion with restriction enzymes *Bam*Hi, *Kpn*I, *Sal*I, and *Sma*I, the DNA fragments were separated by electrophoresis through agarose gel and transferred by blotting to nitrocellulose membrane. The gp120 DNA sequences to be used as a probe were labeled with horseradish peroxidase and hybridization and detection were carried out as described for the Enhanced Chemiluminescence Detection System. A 1.4 kb *Bam*HI band corresponding to the 1.4 kb gp120 sequences flanked by *Bam*HI sites in the vaccine vector pAD.ENV.ND1 used to generate this recombinant adenovirus was not detected in these isolates. Instead, the *Bam*HI Ad2 fragment that hybridized to the gp120 DNA probe was larger than the 9.4 kb lambda *Hind* III marker (data not shown) indicating that a *Bam*HI site in the original vaccine vector had been modified or that a portion of the vector sequences had been deleted. Hybridizing bands were detected in positions roughly corresponding in size to the *Kpn*I A fragment, the *Sal*I A fragment, and the *Sma*I C fragment of wild type Ad2 (Figure 1). These Ad2 restriction enzyme fragments either include (*Kpn*I, *Sal*I) or flank (*Sma*I) the E3 region of Ad2. The results were interpreted as indicating that the gp120 sequences contained in the vaccine vector were inserted into this region of wild type adenovirus. Additional hybridizing bands in the *Kpn*I

digest may reflect the presence of a KpnI site in the original vector construct. The presence of bands in the ethidium bromide stained gel used for Southern blotting (data not shown) that correspond in size to published values for wild type Ad2 indicated that these isolates do not represent pure stocks of recombinant virus.

G4A isolates A11, G1 and G6 were repurified by a second plaque isolation in which 293 cells were infected with 10^{-4} - 10^{-6} dilutions of culture medium from the 96-well plates used for screening. Individual plaques were "picked" and the agar plugs used as inoculum for 293 cells grown in 96-well plates. The lysates were screened for the presence of HIV envelope sequences by hybridization to a digoxigenin-labeled gp120 probe. The percentage of plaques that produced a positive hybridization signal (Table 4) increased significantly from the first to the second plaque purification (3% versus 50% positive). Therefore, the second plaque purification resulted in a significant enrichment in the ratio of recombinant to wild type virus.

The culture medium from two of the gp120 hybridization positive wells of cells infected with second plaque isolates (A11-E1 and -F3, G1-A8 and -H12, and G6-A1 and -B2) was used as inoculum to prepare recombinant virus stocks in 293 cells. The stocks ranged in titer from 9.6×10^{8} - 1.7×10^{-9} PFU/ml. Low molecular weight DNA was extracted from a portion of the infected cells and digested with three restriction enzymes, BamHI, KpnI and SalI. The DNA fragments were separated by electrophoresis through an agarose gel, transferred to nitrocellulose membrane and hybridized to gp120 and SV40 T Ag probes labeled with horseradish peroxidase. Hybridization and detection were carried out as described for the Enhanced Chemiluminescence Detection System. On the basis of the size of the hybridizing bands (Figure 2), the insertion of gp120 sequences in the recombinant G1 and G6 isolates was localized to the 14 kb BamHI, 7.7 kb KpnI and 19 kb SalI A fragments at the right end of the Ad2 genome as would be expected if recombination had occurred into the Ad early region 3. Recombinant viruses G1 and G6 did not hybridize with the SV40 T Ag probe. Therefore, we conclude that the SV40 sequences have been deleted from these viruses. In contrast, similar Southern blot hybridization analysis of the two A11 isolates derived from the second plaque purification (data not shown) indicated that this recombinant virus was derived by insertion of the gp120 sequences into the left end of the Ad2 genome; perhaps, via the Ad major late promoter sequences present in the vaccine plasmid pAD.ENV.ND1. Based upon the size of bands hybridizing to the gp120

probe, the recombination event was localized to the Bam HI and KpnI B bands and the SmaI E band in the Ad early region 1B between nucleotides 2045 and 3931.

To determine if cells infected with these recombinant virus isolates produced the gp120 envelope protein, 293 cells grown in chamber slides were infected with 10^{-2} dilutions of either the six isolates or wild type Ad2. At 24 hours PI, viral cytopathology was observed in 50% of the cells and the infected monolayers were fixed for immunocytochemistry. The virus-infected cells were analyzed by immunoperoxidase staining reactions with four primary antibodies: two anti-HIV envelope monoclonal antibodies and two polyclonal antibodies. Specific staining was observed only with the rabbit anti-gp120 serum (Figure 3). Cells infected with isolates A11-F3 and G6-A1 did not react with any of the antibodies tested.

3. Generation of a Recombinant HIV env160/ Adenovirus 2

In an attempt to facilitate selection of recombinant adenoviruses, we have devised a new procedure (Figure 4A) for obtaining recombinants that involves transfecting cells with fragments of Ad2 DNA rather than the full-length wild type adenovirus genome. The components are: 1) an AseI fragment of Ad2 DNA extending from the left end of the Ad2 genome to the AseI site at 89 map units; 2) a vaccine vector containing HIV envelope sequences flanked by adenovirus E3 sequences; and 3) the right-most XbaI C fragment from Ad2 (88.5 - 100 map units). The 31.9 kb AseI DNA fragment was isolated by electrophoresis through 0.3% agarose gel and electroelution. The 4.1 kb XbaI C fragment was similarly isolated by electrophoresis through 0.7% agarose gel. In the initial attempt to obtain a recombinant adenovirus containing gp160 sequences, 293 cells grown in 6-well plates were transfected with a calcium phosphate coprecipitate of 1 ug plasmid pAd.ENV(160) linearized with AatII, 1 ug of Ad2 AseI DNA fragment and 0.75 ug of Ad2 XbaI C fragment. As controls, 293 cells were transfected with plasmid pAD.ENV(160) DNA only, with a mixture of the AseI DNA fragment and the plasmid or with 1 ug of intact Ad2 DNA. Discrete areas of typical adenoviral cytopathology were observed in the 293 cell monolayers transfected with Ad2 DNA within 5 days of exposure to DNA precipitates. However, obvious viral cytopathology was not seen in cells transfected with other DNA or DNA mixtures at 15 days after exposure. At this time, the cells were harvested, subjected to repeated cycles of freezing and thawing to release cell-associated virus and clarified by low speed centrifugation. In a blind passage, dilutions of the super-

natant culture medium were used to inoculate monolayers of 293 cells that were subsequently monitored for viral cytopathology for an additional two weeks. No recombinant viruses were obtained from this transfection.

The plasmid pIPA was constructed by a modification of the method of Berkner and Sharp (13) to provide a readily available source of the 4.1 kb XbaI Ad2 DNA fragment for use in generating recombinant adenoviruses. In order to clone the Ad2 DNA termini, it was necessary to remove the 55K-terminal protein but leave the Ad terminal sequence intact. Ad2 DNA was incubated with T4 DNA polymerase and excess dTTP to remove a single nucleotide from the 3′ strand (Figure 5). S1 nuclease was used to remove the protruding 5′-dCMP and covalently bound 55K protein and BamHI linkers were added with T4 ligase. The Ad2 DNA with linkers attached was digested with BamHI and XbaI and the fragments separated by electrophoresis through agarose gel. The 4.1 kb XbaI fragment was recovered from the gel and ligated with pBR327 digested with BamHI and NheI to yeild pIPA. A restriction enzyme map and schematic diagram of pIPA is shown in Figure 6.

A diagram of the use of pIPA for generation of recombinant adenoviruses is shown in Figure 4B. Experimentally, 293 cells in 6-well plates were transfected with 1 ug of the Ad2 AseI DNA fragment, 1.5 ug pIPA linearized with ClaI, and 1.5 ug pAD.ENV (HIV gp120) linearized with SspI or 1.5 ug pAD.ENV(160) linearized with SspII plus 10 ug Lipofectin Reagent. Cells were transfected with 4 ug of intact wild type Ad2 DNA as a control. Within 2 days of exposure to Ad2 DNA, viral cytopathology was evident and these cells were harvested on day 5. No cytopathology was observed in the cells that had been transfected with the Ad2 AseI fragement and plasmids. After 21 days, the cells were harvested and dilutions of the cell-free supernatant was used for blind passage on 293 cell monolayers. After one week in culture, low molecular weight DNA was extracted from these cells by the method of Hirt (61). The DNA was digested with Bam HI, separated by electrophoresis through 0.7% agarose and transferred to nitrocellulose membrane. The blotted DNA was hybridized to both the gp120 DNA probe and the Ad2 AseI DNA fragment labeled with horseradish peroxidase using the ECL Gene Detection System. A positive signal was not observed with either probe leading us to conclude that recombinant adenovirus had not been generated in this transfection.

Although the majority of progeny virus obtained from a transfection using these three components should be recombinants, the frequency of this event will be very low because multiple recombination events are

required to obtain the desired adenovirus. In an attempt to increase the probability of obtaining a recombinant virus by decreasing the number of recombinant events necessary to generate this virus, a second plasmid, pUBA, was constructed. For this construct, pAD.ENV(160) was digested with XmnI and an approximately 7570 base pair fragment including the E3 flanking regions, HIV gp160 sequences, the Ad major late promoter, the ori gene from pBR322 (pUC19) and a portion of the pBR322 bla gene (pUC19) was isolated. Plasmid pIPA was also digested with XmnI to yeild a 5800 base pair fragment containing the right-most XmnI fragment of Ad2 and the remaining portion of the pBR322 bla gene. Ligation of these fragments in the proper orientation yielded plasmid pUBA. A restriction enzyme map and schematic diagram of pUBA is shown in Figure 7. The use of this plasmid for generation of recombinant HIV gp160/Ad2 is shown schematically in Figure 4C. Transfection experiments using this plasmid have not yet been carried out.

4. Construction of "Cassette" Vaccine Vector

Each of the vaccine vectors used in this project was constructed independently via a series of intermediate plasmid constructs. The development of vaccine vectors would be greatly facilitated by the availability of a "cassette" vector into which sequences encoding additional potential HIV-1 antigens or antigenic peptides, antigenic proteins from various HIV-1 isolates or antigens derived from other retroviruses could be readily inserted. We propose to construct two cassette vaccine vectors consisting of HIV gp120 or gp160 sequences plus and minus the Ad major late promoter flanked by Ad E3 sequences to facilitate recombination into wild type Ad2. In the case of the construct minus the promoter, transcription of HIV sequences would be assumed to be initiated from the endogenous wild type E3 promoter early in virus infection and from the endogenous major late promoter late in infection. The HIV envelope sequences in the cassette vaccine vector will be flanked by unique BamHI and XhoI sites to make removal of the envelope sequences and insertion of another DNA fragment of interest relatively easy. These restriction enzyme sequences were selected because of the under-representation of BamHI and XhoI sites in HIV-1 isolates.

Development of the cassette vaccine vectors was begun by constructing a pBR327 plasmid that lacks the BamHI site. To do this, pBR327 was digested with Bam HI and the recessed 3' termini were filled-in using the Klenow fragment of DNA polymerase followed by religation with T4 ligase. A 4752 base pair fragment encompassing the Ad E3 region was obtained by digesting plasmid

pUC.AD2 with EcoRV. This fragment was ligated into the Bam⁻ pBR327 plasmid that had been digested with Eco RV. The resultant plasmid construct provides the Ad sequences necessary for recombination of the vaccine vector into the Ad viral genome. Restriction enzyme maps and a schematic diagram of plasmid pB⁻ (pBR327 with the BamHI site deleted) and pBA (pB⁻ containing Ad E3 sequences) are shown in Figures 8 and 9, respectively.

F. Conclusions

The procedure originally proposed for generation of recombinant HIV gp120/Ad2, i.e. transfecting cells with wild type Ad2 DNA and a plasmid containing the HIV gp120 sequences, yields mostly wild type progeny making screening for recombinant viruses a monumental task. The problem of screening coupled with the instability of the recombinant virus and the generation of replication defective recombinant adenoviruses in which the gp120 sequences were introduced into regions other than early region 3 led us to consider alternative strategies for construction of the HIV gp120/Ad2. Although screening for recombinant viruses continued this year in an attempt to obtain a plaque-purified isolate that produced significant quantities of the gp120 protein, a portion of our time was spent in developing methods for generation of recombinant Ad2 that would yield no or very few wild type viruses. In addition, we also began work on the construction of "casette" vaccine vectors that would much simplify the insertion of foreign DNA sequences into the plasmid used for ultimate recombination into the adenovirus genome.

G. Recommendations

It is essential to alter the method used for generation of the recombinant adenoviruses in order to decrease the magnitude of screening required to isolate potential replication-competent recombinant viruses. We also recommend that any further research to develop a recombinant adenovirus for use as a human vaccine be carried out with a less common adenovirus serotype than 2; perhaps, adenovirus type 16, 20 or 25 would be suitable candidates. In addition, we recommend that the SV40 promoter and T Ag sequences be deleted from vaccine vector constructs as these sequences may be responsible for some of the observed instability of the recombinant virus. Because deletion of these sequences would preclude effective safety and efficacy-testing of the recombinant HIV gp120/Ad2 in monkeys, we recommend that similar recombinant simian adenoviruses be constructed using either the SIV or HIV-2 env gene for testing in macaques or Cercopithecus, respectively. This would provide a valid animal model system in which

to test the efficacy of live adenovirus vaccines with results that should be applicable to the human adenovirus equivalent.

TABLE 1

Retroviruses Infection in Wild Cercopithecus

Number	Abbott			Quick Test
2438	++	1		Negative
2443	+			HIV-I & HIV-2+
2444	N.D.			HIV-1+
2447	N.D.			HIV-1+
2448	+	1		Negative
2449	N.D.			HIV-1+
2452	Weak			Intertermitant
2456	N.D.			HIV-1+, HIV-2+
2461	N.D.			Negative
2475	+	1		Negative
2485				HIV-1+
2489	-			HIV-1+
2490	+	1		HIV-2+
2491				Negative
2524				Negative
2533	+	1		Negative
2538	N.D.			HIV-1+, HIV-2+
2539	N.D.			HIV-1+, HIV-2+
2543	N.D.			HIV-2+, HIV-1+
2545	N.D.			HIV-1+
2547	+	1		Negative
2549	-			Negative
2555	-			Negative
2554	-			Negative
	dual	5/24	20.8%	
	HIV-1	11/24	45.8%	1 uncertain
	HIV-2	1/24	4.2%	

TABLE 2

Isolation of Recombinant HIV gp120/Adenoviruses by Limited Dilution Screening

Original Transfectant	Vaccine Plasmid[A]	% Wells with CPE[B]	Hybridization Positive[C] Wells per Total Wells	% Hybridization Positive Wells per Wells with CPE
J3A	pAD.ENV.ND1	40.1	1/1012	0.25%
G4A	pAD.ENV.ND1	94.2	1/1012	0.1%
H6B	pAD.ENV	68.2	2/1012	0.3%

[A]Plasmid used in transfection to obtain recombinant virus.

[B]CPE = cytopathology

[C]Cell wells that give a positive hybridization signal with a digoxigenin-labeled gp120 probe.

TABLE 3

Isolation of Recombinant HIV gp120/Adenoviruses by Plaque Purification: First Purification

Isolate	Titer: PFU/ml[A]	Number of Plaques Tested[B]	Hybridization Positive[C] Wells
J3A	$1.2 \cdot 10^8$	184	0
G4A	$7.7 \cdot 10^8$	92	3
H6B-A	$7.1 \cdot 10^8$	92	0
H6B-C	$1.3 \cdot 10^8$	184	0

[A]PFU = Plaque Forming Units

[B]Individual plaques, as agar plugs, were used as inoculum to infect a well of 293 cells in 96-well plates.

[C]Cell wells that gave a positive hybridization signal with a digoxigenin-labeled gp120 probe.

TABLE 4

Isolation of Recombinant HIV gp120/Adenoviruses by Plaque Purification: Second Purification

Isolate	Titer: PFU/ml[A]	Number of Positive Plaques per Total Plaques Tested[B]	% Positive Plaques
G4A-G6	1.58×10^6	46/93	50%
G4A-G1	3.16×10^5	55/96	57%
G4A-A11	4.75×10^6	38/96	40%

[A]PFU = Plaque Forming Units

[B]Individual plaques, as agar plugs, were used as inoculum to infect a well of 293 cells on 96-well plates. Lysed cells were transferred to nylon membrane as dot blots and hybridized with a digoxigenin-labeled gp120 probe.

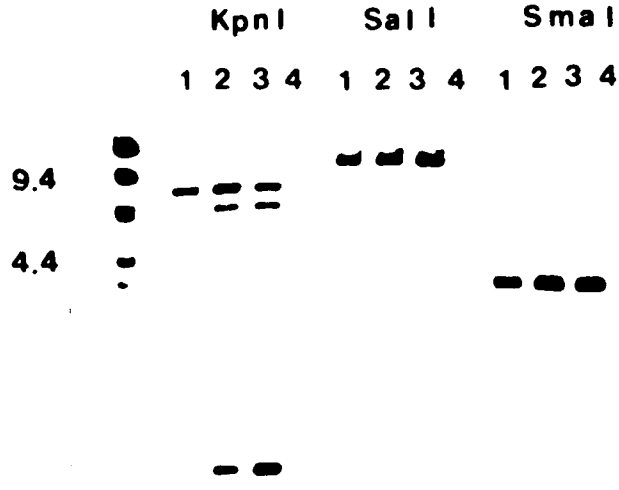
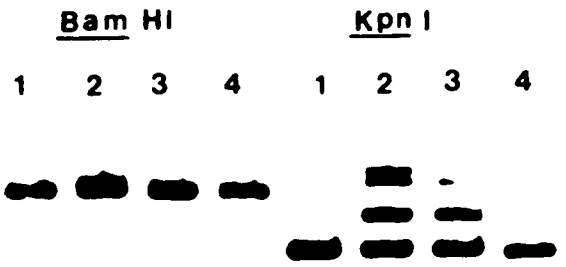


Figure 1. Southern blot hybridization of DNA from recombinant adenovirus isolates digested with KpnI, SalI, and SmaI. DNA (2 ug) isolated from 293 cells infected with recombinant virus plaque isolates A11(1), G1(2) and G6(3) was digested with restriction enzymes, electrophoresed in an agarose gel, transferred to a nitrocellulose membrane and probed by the enhanced chemiluminescence method with HIG gp120 sequences labeled with horseradish peroxidase. Lane 4 contains 1 ug of wild type Ad2 DNA. The left-most lane contains λ-HindIII standards hybridized to horseradish peroxidase-labeled λ-HindIII DNA fragments.



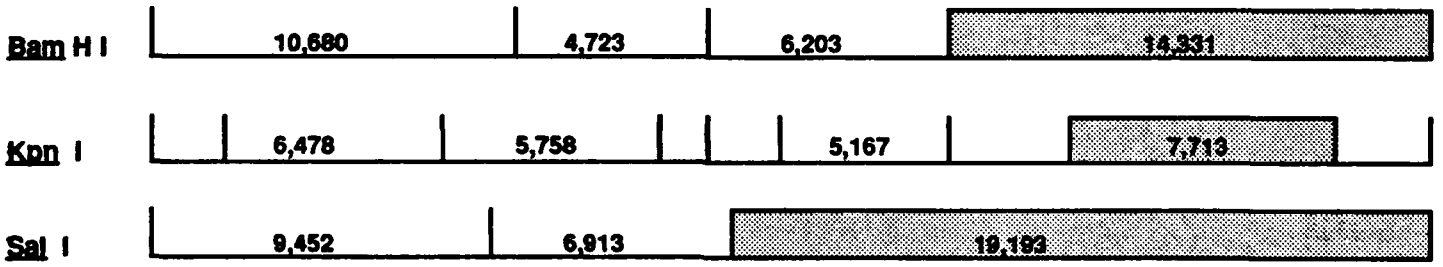


Fig. 2. Southern blot hybridization of DNA from recombinant adenovirus isolates G1 (Lanes 1 and 2) and G6 (Lanes 3 and 4) digested with Bam HI and Kpn I. DNA (2 μg) isolated from 293 cells infected with two plaque isolates of G1 and G6 was digested with restriction enzymes, electrophoresed in an agarose gel, transferred to a nitrocellulose membrane and probed by the enhanced chemiluminescence method with HIV gp 120 sequences labeled with horseradish peroxidase.

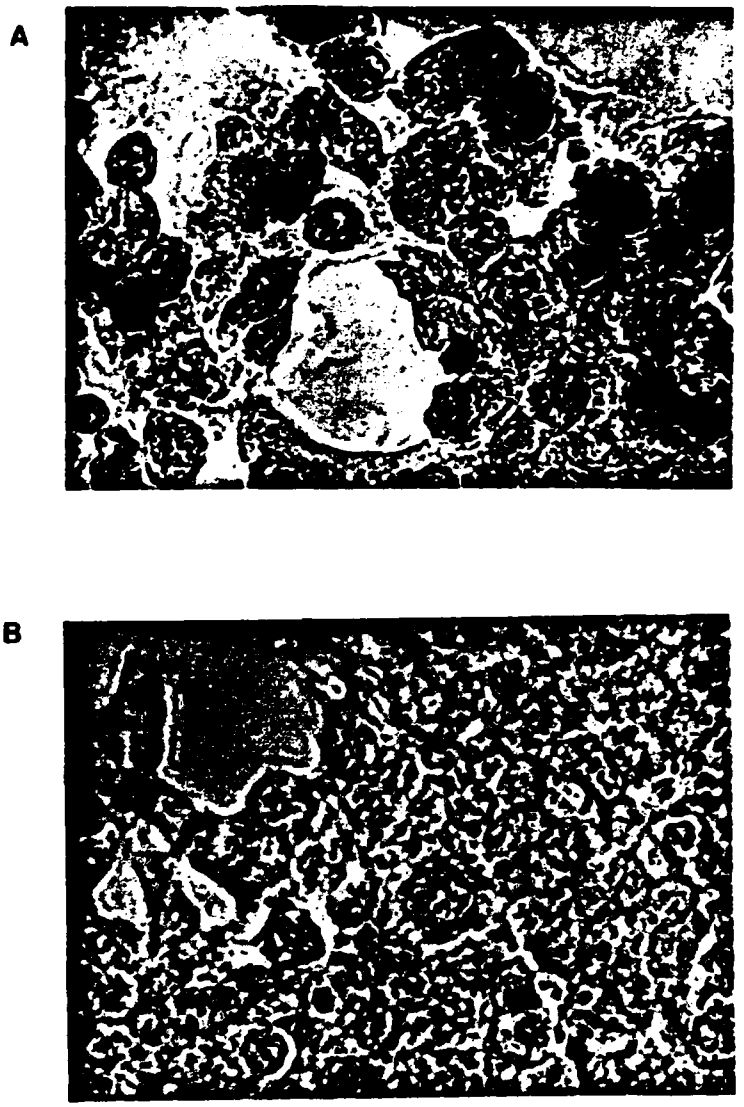
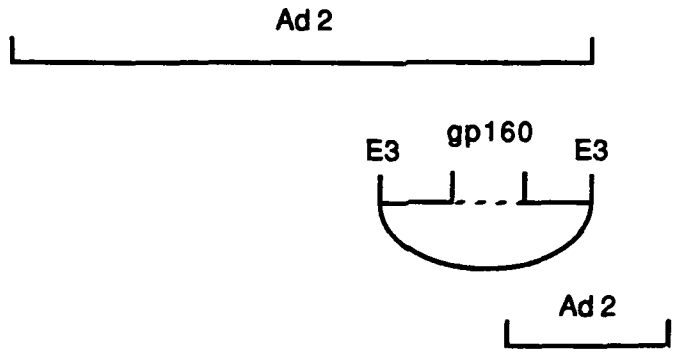
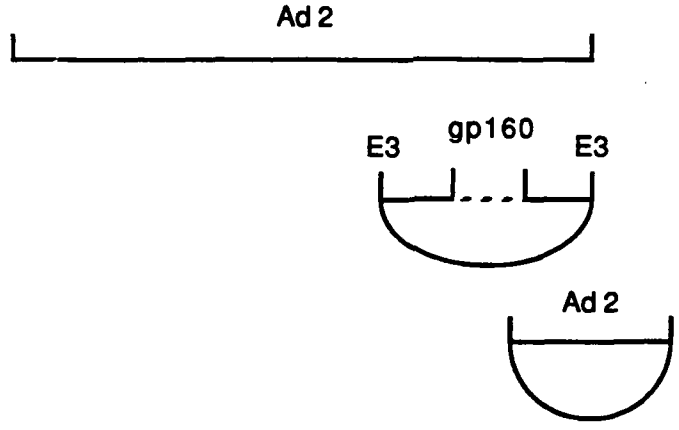


Figure 3 Accumulation of HIV-1 gp120 in 293 cells infected with recombinant HIV-1 gp120/Ad2. 293 cells infected with isolate G1-A8 (A) or wild type Ad2 (B) were fixed in methanol-acetic acid and acetone at 24 hour PI. The cells were stained using a rabbit anti-gp120 as primary antibody and peroxidase-conjugated swine anti-rabbit immunoglobulins as second antibody. Antibody binding was visualized with hydrogen peroxide and 3,3',5,5' - tetramethylbenzidine.

A.



B.



C.

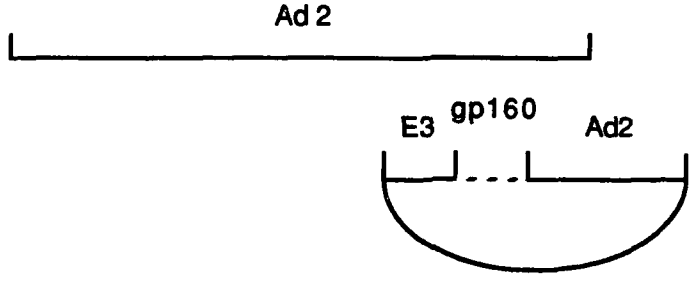


Fig. 4. Construction of recombinant adenoviruses. Cells are cotransfected with: A) an AseI fragment of Ad2 DNA, a vaccine vector containing HIV gp160 sequences and the Ad2 XbaI C fragment; or B) the Ad2 AseI fragment and vaccine vector plus plasmid pIPA containing the Ad2 XbaI fragment; or C) the Ad2 AseI fragment plus plasmid pUBA containing both gp120 sequences and the Ad2 XbaI fragment.

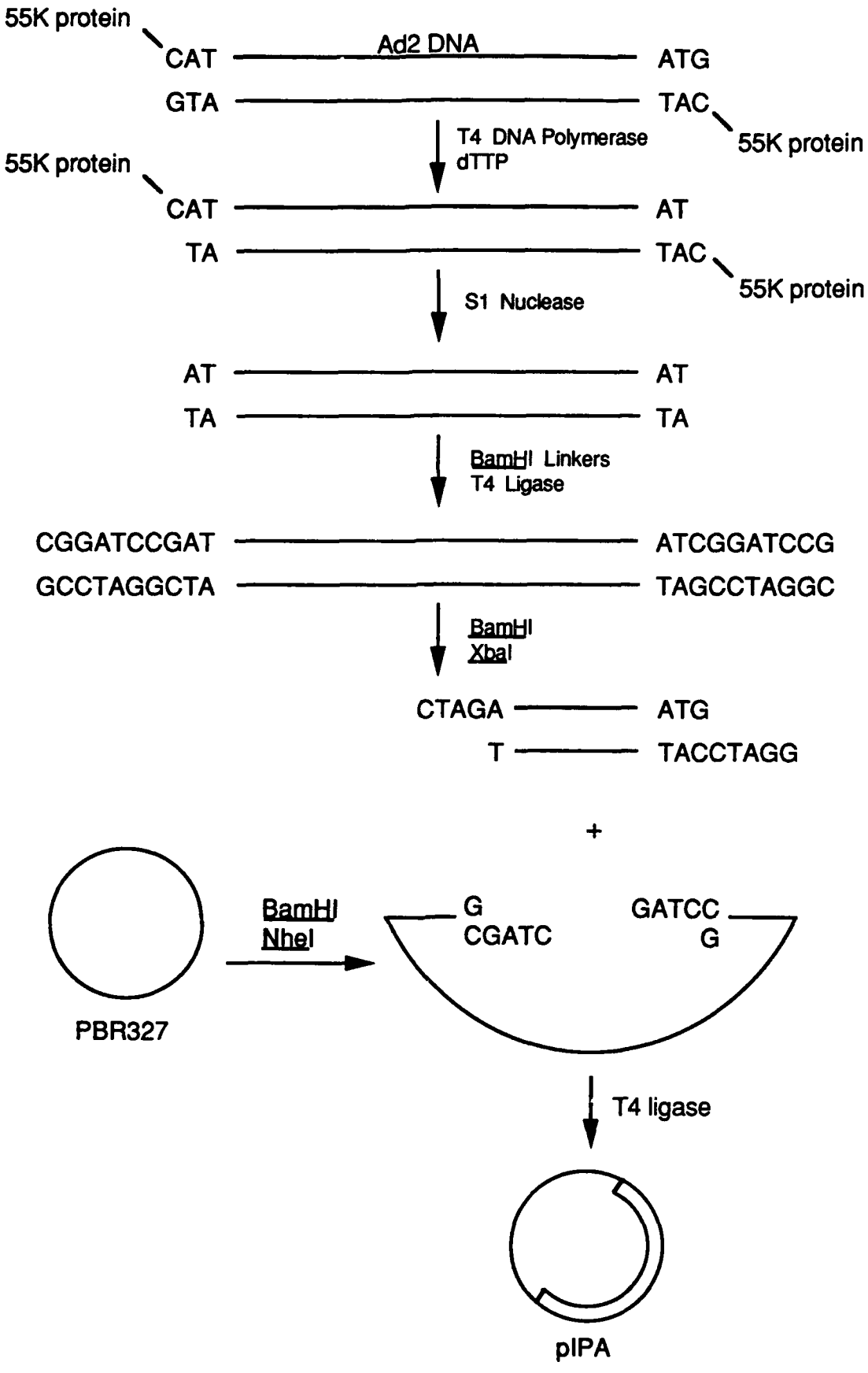
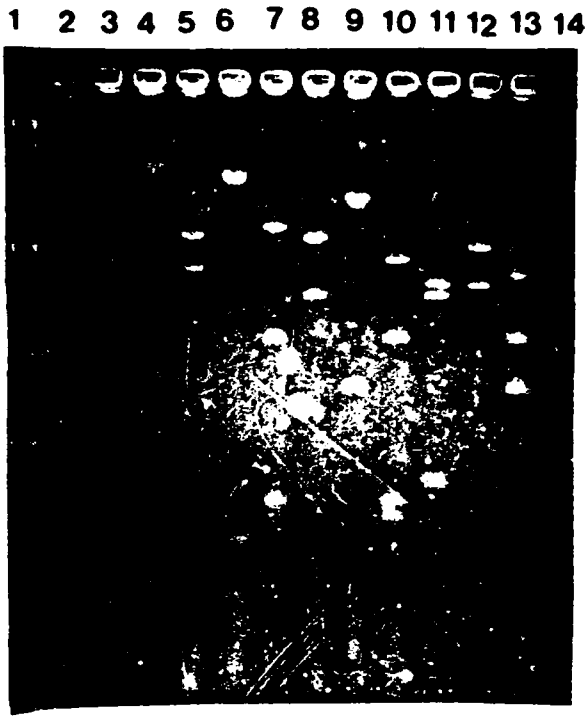


Fig. 5. Construction of plasmid pIPA containing right-most Xba1 fragment of Ad2

A



B

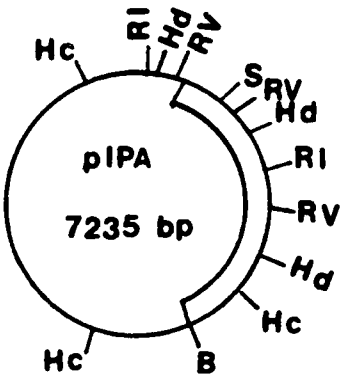
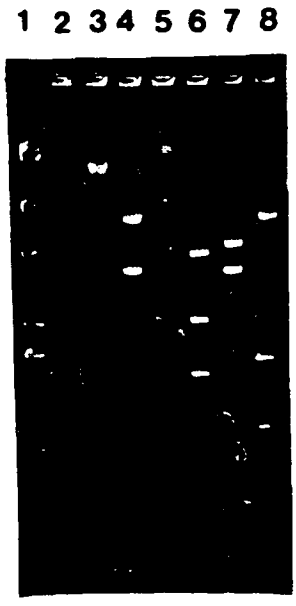


Figure 6. Analysis of plasmid pIPA. A. Plasmid DNA was digested with restriction endonucleases and fractionated on a 1.2% agarose gel. Lane 1, λ*Hin*dIII + ϕX *Hpa*I DNA standard; 2, uncut; 3, *Bam*HI; 4, *Stu*I; 5, *Bam*HI + *Stu*I; 6, *Eco*RI; 7, *Hinc*II; 8, *Hin*dIII; 9, *Eco*RV; 10, *Stu*I + *Hinc*II; 11, *Bam*HI + *Hin*dIII; 12, *Bam*HI + *Eco*RI; 13, *Bam*HI + *Eco*RV; 14, *Eco*RI + *Hinc*II. The 300 bp *Eco*RV band (Lanes 9 and 13) and the 465 bp *Eco*RI + *Hinc*II band (Lane 14) are not visible on this gel. B. Structure of pIPA with relevant restriction endonuclease cleavage sites: B, *Bam*HI; S, *Stu*I ; RI, *Eco*RI; RV, *Eco*RV; Hc, *Hinc*II; Hd, *Hin*dIII.

A



B.

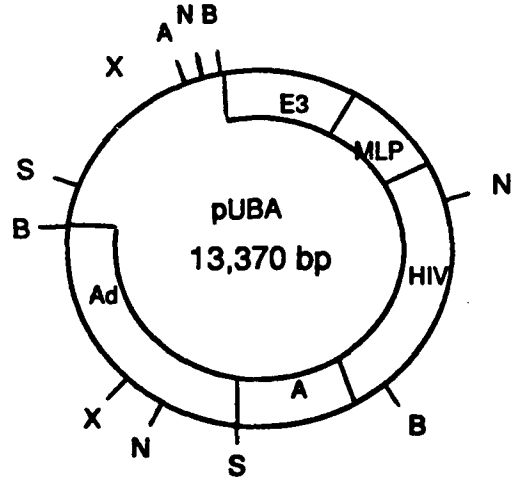


Fig. 7. Analysis of plasmid pUBA. A. Plasmid DNA was digested with restriction endonucleases and fractionated on a 1% agarose gel. Lane 1, Lambda HindIII and øx HpaI DNA standard; 2, uncut; 3, AatII; 4, SalI; 5, XmnI; 6, BamHI; 7, EcoRI; 8, NdeI. B. Structure of pUBA with relevant restriction endonuclease cleavage sites: A, AatII; S, SalI; X, XmnI; B, BamHI; N, NdeI; E3, Ad2 early region 3; MLP, Ad2 major late promoter; HIV, HIV gp 160; A, poly A addition signal; Ad, left end of Ad2.

A

B.

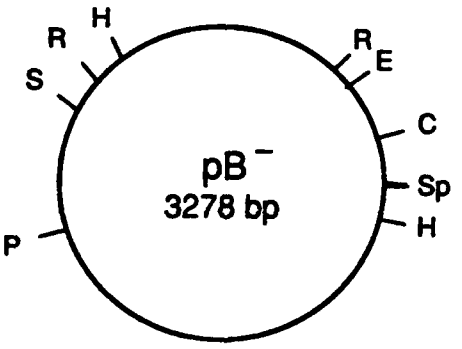
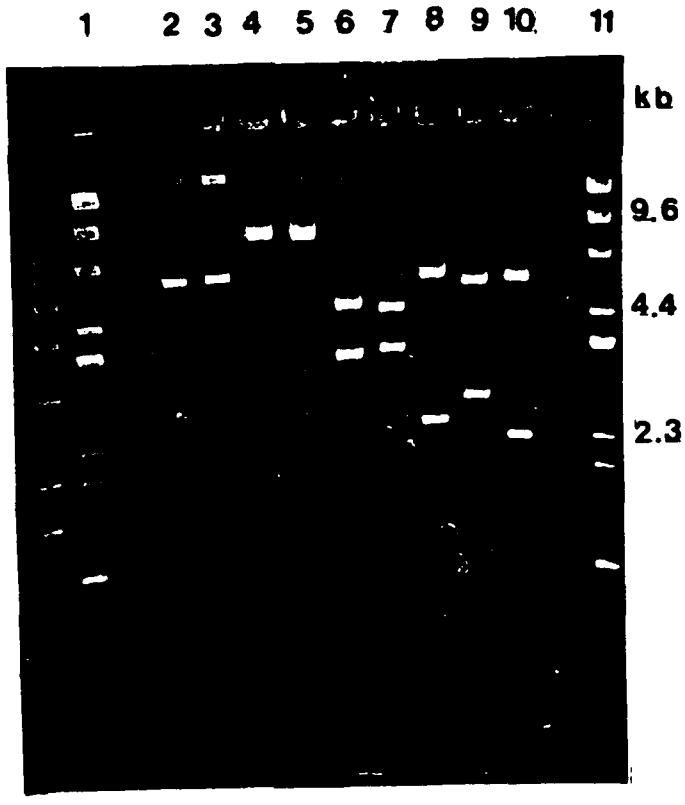


Fig. 8. Analysis of plasmid pB⁻. A. Plasmid DNA was digested with restriction endonucleases and fractionated on a 1% agarose gel. Lanes 1 and 10, Lambda HindIII and øx HpaI DNA standard; 2, uncut; 3, BamHI; 4, ClaI; 5, EcoRV; 6, HincII (incomplete digest); 7, RsaI; 8, ScaI + SphI (incomplete digest); 9, PstI and SphI. B. Structure of pB⁻ with relevant restriction endonuclease cleavage sites: C, ClaI; E, EcoRV; H, HincII; R, RsaI; S, ScaI; P, PstI; Sp, SphI.

A



B.

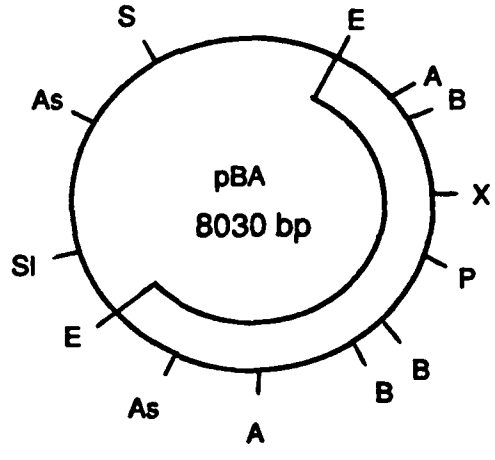


Fig. 9. Analysis of plasmid pBA. A. Plasmid DNA was digested with restriction endonucleases and fractionated on a 1% agarose gel. Lanes 1 and 11, Lambda HindIII and øx HpaI DNA standard; 2, *uncut*; 3, BamHI; 4, EcoRV; 5, ApaI; 6, PvuII + ScaI; 7, AseI; 8, SalI + XhoI; 9, BglII. B. Structure of pBA with relevant restriction endonuclease cleavage sites: E, EcoRV; A, ApaI; P, PvuII; S, ScaI; As, AseI; Sl, SalI; X, XhoI; B BglII.

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